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Each flying fox on its own branch: A phylogenetic tree for *Pteropus* and related genera (Chiroptera: Pteropodidae)

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ABSTRACT

Pteropodidae is a diverse Old World family of non-echolocating, frugivorous and nectarivorous bats that includes the flying foxes (genus *Pteropus*) and allied genera. The subfamily Pteropodinae includes the largest living bats and is distributed across an immense geographic range from islands in East Africa to the Cook Islands of Polynesia. These bats are keystone species in their ecosystems and some carry zoonotic diseases that are increasingly a focus of interest in biomedical research. Here we present a comprehensive phylogeny for pteropodines focused on *Pteropus*. The analyses included 50 of the ~63 species of *Pteropus* and 11 species from 7 related genera. We obtained sequences of the cytochrome b and the 12S rRNA mitochondrial genes for all species and sequences of the nuclear RAG1, vWF, and BRCA1 genes for a subsample of taxa. Some of the sequences of *Pteropus* were obtained from skin biopsies of museum specimens including that of an extinct species, *P. tokudae*. The resulting trees recovered *Pteropus* as monophyletic, although further work is needed to determine whether *P. personatus* belongs in the genus. Monophyly of the majority of traditionally-recognized *Pteropus* species groups was rejected, but statistical support was strong for several clades on which we based a new classification of the *Pteropus* species into 13 species groups. Other noteworthy results emerged regarding species status of several problematic taxa, including recognition of *P. capistratus* and *P. ennisae* as distinct species, paraphyly of the *P. hypomelanus* complex, and conspecific status of *P. pelewensis pelewensis* and *P. p. yapensis*. Relationships among the pteropodine genera were not completely resolved with the current dataset. Divergence time analysis suggests that *Pteropus* originated in the Miocene and that two independent bursts of diversification occurred in the Pleistocene in different regions of the Indo-Pacific realm.

1. Introduction

Old World fruit bats (family Pteropodidae) are a diverse group of non-echolocating bats that inhabit tropical regions in Africa, Asia, Australia, and many Pacific islands. *Pteropus* Brisson, 1762 (flying foxes) is the type and most speciose genus, including approximately 63 species, or about a third of the nearly 200 species in the family (Simmons, 2005). Flying foxes and their close relatives (tribe Pteropodini *sensu* Bergmans, 1997) include the largest living bats, reaching 1.5 kg in *Pteropus vampyrus* and *Acerodon jubatus* (Kunz and Pierson, 1994), fairly close to the theoretical upper body mass limit estimated for bats as flying mammals (about 2 kg; U. Norberg, pers. comm.). Because of their ecological habits (e.g., roosting in trees, inhabiting coastal areas, and feeding from fruit) and because they are a source of food for some human populations, many *Pteropus* species come into indirect or direct contact with humans and livestock (Mickleburgh et al., 1992, 2009). In recent years *Pteropus* species have been increasingly recognized as natural reservoir hosts for a number of important zoonotic diseases including Henipaviruses and Paramyxoviruses (e.g., Halpin et al., 2011; Drexler et al., 2012; Epstein et al., 2008; Hahn et al., 2014).

The distribution of *Pteropus* covers an immense range of territories across the Indian and Pacific Oceans, from Pemba and Mafia Islands in East Africa, to the Cook Islands of Polynesia (Simmons, 2005; Helgen et al., 2009). A minority of species occupy extensive areas on continents and large islands, specifically Madagascar

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(*Pteropus rufus*), India and Southeast Asia (e.g., *P. medius* [formerly known as *P. giganteus;* see Mlíkovsky, 2012], *P. vampyrus*), New Guinea (e.g., *P. neohibernicus*) and Australia (e.g., *P. scapulatus, P. poliocephalus*). However, *Pteropus* is a predominantly insular taxon, with most species occurring in islands and coastal areas (Helgen et al., 2009). As such, flying foxes play key roles in the ecology of those habitats as seed and pollen dispersers (Fujita and Tuttle, 1991; Entwistle and Corp, 1997; Smith and Leslie, 2006). For instance, flying foxes in the Samoa archipelago interact with 59% of the forest tree species that provide fruit or flower resources (Banack, 1998). *Pteropus* species are capable of flying up to 50 km in a single night, making it possible for them to effectively disperse seeds and pollen over large areas (Mickleburgh et al., 1992). Vulnerability to extinction within this insular specialist taxon is reflected in the fact that 6 insular or coastal species of *Pteropus* are considered to have become extinct over the past 150 years (*P. tokudae* of Guam, *P. pilosus* of Palau, *P. subniger* of the Mascarenes, *P. brunneus* of coastal NE Australia, and *P. allenorum* and *P. coxi* of Samoa; see Helgen et al., 2009), and as many as 37 species are currently included in a formal category of extinction risk by the IUCN (from Near Threatened to Critically Endangered; IUCN, 2013 and citations therein).

Andersen (1912) produced the historically most influential classification of Old World fruit bats, a monographic tome of over 800 pages of which over half were devoted to *Pteropus*. He grouped the *Pteropus* species that he considered valid into 17 species groups (Andersen, 1912). Pelage patterns and features such as robustness of the skull, mandible and dentition figured prominently among the systematic characters used to allocate species to groups (Andersen, 1912). While some of Andersen's (1912) species groups were small groups that included extremely similar taxa from adjacent geographic regions (e.g., the *vampyrus* species group), this was not always the case. O'Brien et al. (2009) and others have noted the general lack of correspondence between species groups and geographic distribution patterns of *Pteropus* species. Nevertheless, in the absence of subsequent comprehensive assessments, most authors to date have followed the general outline of Andersen's (1912) groups (e.g., Koopman, 1993). Similarly, most species described since Andersen's (1912) publication have been allocated to one of the groups he recognized (e.g., *Pteropus fundatus* Felten and Kock, 1972 was assigned to Andersen's *chrysoproctus* species group). No major modifications were made to Andersen's (1912) scheme either at the genus level or within species groups until very recently. Giannini et al. (2008) revalidated *Desmalopex* Miller, 1907, a taxon treated as a junior synonym of *Pteropus* by Andersen (1912) on the basis of perceived affinities with his *pselaphon* species group. *Desmalopex* is now known to include two Philippine endemics, *D. leucopterus* (Luzon, Catanduanes) and *D. microleucopterus* (Mindoro; Esselstyn et al., 2008), which form a clade that falls well outside *Pteropus* within Pteropodini (Giannini et al., 2008).

Even with the removal of *Desmalopex*, *Pteropus* may not be monophyletic (see Giannini et al., 2008). Closely related pteropodine genera, most notably *Acerodon* but also *Neopteryx* and *Stylocteniu*m, share many similarities with some species of *Pteropus* (see Andersen, 1912) and may well be phylogenetically nested within *Pteropus*. Further, several species currently included in *Pteropus* are as morphologically distinctive as *Desmalopex* and may belong outside the core *Pteropus* clade. All of this is difficult to assess at present because the monophyly of most *Pteropus* species groups has been neither comprehensively nor extensively tested. A recent contribution has shown that none of the four *Pteropus* species groups traditionally recognized in the Indian Ocean are monophyletic (O'Brien et al., 2009). Instead, three alternative groups were recovered, two of which were nested within an expanded *vampyrus* species group (O'Brien et al., 2009). The western Indian Ocean species of *Pteropus*, with the exception of some subspecies of the complex taxon *P. hypomelanus*, were found to belong in a single clade with affinities to the *vampyrus* species group. In total, recent studies including Giannini et al. (2008), Esselstyn et al. (2008) and O'Brien et al. (2009) have questioned the monophyly of all the groups so far included in the analyses, or one-third of Andersen's (1912) species groups (i.e., the *vampyrus*, *niger*, *subniger*, *molossinus*, *livingstonii*, and *pselaphon* species groups), underlining the need for a comprehensive phylogenetic overview of the classification of *Pteropus*.

Here we address three key aspects of flying fox evolution: 1. monophyly of *Pteropus*; 2. phylogenetic support (or lack thereof) for the previously-proposed *Pteropus* species groups; and 3. age of the major clades within *Pteropus*. To this end we compiled a fragmented but nearly comprehensive molecular dataset, including both new sequences (many from museum specimens) and sequences from previous studies, which together comprise the largest taxonomic sample to date for *Pteropus*. Our sample includes representatives from all but 3 of the traditional species groups as well as all the relevant outgroups. Results of analyses of these data highlight the need for a complete revision of *Pteropus* and reveal key aspects of the evolutionary history of flying foxes.

2. Methods

2.1. Samples

To study the relationships among *Pteropus* species we obtained 42 tissue samples, representing 24 species, from various institutions and individuals (see Supplementary Table S1). Additionally, we collected and analyzed 48 skin samples from the collections of the American Museum of Natural History, New York (AMNH) and the National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM), representing 28 species (Supplementary Tables S1 and S2). Skin samples were excised from the wings using 3 mm punches. One specimen (AMNH 274462) had no skin and therefore a sample of dry muscle tissue attached to the skeleton was collected instead. When possible, we chose to sample specimens with confirmed collection localities. In order to increase our taxonomic sampling, we also gathered all *Pteropus* sequences available in GenBank (Supplementary Table S3). Some species were represented by multiple sequences, in some cases obtained from different sources (tissue, skin, GenBank). We first performed preliminary phylogenetic analyses using all available sequences to try to detect contamination and misidentified samples. We then assembled a matrix with representative sequences (the longest for each species) of 50 *Pteropus* species, most of which had never been included in a molecular phylogenetics study. One of the museum skin samples we analyzed represents *Pteropus tokudae*, an extinct Guam endemic last recorded in the 1960s or 70s (Bonaccorso et al., 2008). Our samples included representatives of 15 of the 18 *Pteropus* species groups currently recognized (Simmons, 2005).

In addition to the *Pteropus* species mentioned above, we sequenced samples or obtained published sequences for 7 genera closely related to *Pteropus*: *Melonycteris* (2 spp.), *Desmalopex* (2 spp.), *Acerodon* (2 spp.), *Styloctenium* (1 sp.), *Pteralopex* (2 spp.), *Mirimiri* (1 sp.), and *Neopteryx* (1 sp.) (Supplementary Tables S1 and S3). For the outgroup, we included three species of the genus *Nyctimene*. All sequences newly obtained for this study were deposited in the GenBank with accession numbers KJ532324–KJ532447.

2.2. Molecular methods

We extracted total genomic DNA using the Qiagen DNeasy tissue kit (QIAGEN). For DNA extraction from museum samples, kit

Table 1
A revised "species group" classification of the genus *Pteropus*, updating Andersen (1912). Species-level taxonomy follows Simmons (2005), as updated by Helgen (2004), Giannini et al. (2008), Helgen et al. (2009), Mlíkovský (2012), Buden et al. (2013), and this paper.

"*personatus*" group	?*personatus* Temminck, 1825	
"*pelagicus*" group	*macrotis* Peters, 1867	
	woodfordi Thomas, 1888	
	mahaganus Sanborn, 1931	
	gilliardorum Van Deusen, 1969	
	molossinus Temminck, 1853	
	pelagicus Kittlitz, 1836	formerly *phaeocephalus*, including *insularis*
	tokudae Tate, 1934*	
"*scapulatus*" group	*scapulatus* Peters, 1862	
"*lombocensis*" group	*lombocensis* Dobson, 1878	
"*livingstonii*" group	*livingstonii* Gray, 1866	
	voeltzkowi Matschie, 1909	
"*vampyrus*" group	*pselaphon* Lay, 1829	
	dasymallus Temminck, 1825	
	pumilus Miller, 1911	
	rodricensis Dobson, 1878	
	vampyrus (Linnaeus, 1758)	
	medius Temminck, 1825	formerly *giganteus*
	lylei Andersen, 1908	
	aldabrensis True, 1893	
	rufus E. Geoffroy, 1803	
	seychellensis Milne-Edwards, 1877	including *comorensis*
	niger (Kerr, 1792)	
"*capistratus*" group	*capistratus* Peters, 1876	
	ennisae Flannery and White (1991)	
	? *temminckii* Peters, 1867	
"*vetulus*" group	*vetulus* Jouan, 1863	
"*samoensis*" group	*nitendiensis* Sanborn, 1930	
	tuberculatus Peters, 1869	
	anetianus Gray, 1870	
	fundatus Felten and Kock, 1972	
	samoensis Peale, 1848	
	rayneri, Gray 1870	
	cognatus Andersen, 1908	
	rennelli Troughton, 1929	
	? *brunneus* Dobson, 1878*	no molecular data
	? *pilosus* Andersen, 1908*	no molecular data
	? *coxi* Helgen et al., 2009*	no molecular data
	? *allenorum* Helgen et al., 2009*	no molecular data
	? *chrysoproctus* Temminck, 1837	no molecular data
"*poliocephalus*" group	*poliocephalus* Temminck, 1825	
"*ornatus*" group	*ornatus* Gray, 1870	
"*griseus*" group	*hypomelanus* Temminck, 1853	
	griseus (E. Geoffroy, 1810)	
	speciosus Andersen, 1908	
	neohibernicus Peters, 1876	
	conspicillatus Gould, 1850	
	alecto Temminck, 1837	including *banakrisi*
	tonganus Quoy and Gaimard, 1830	
	ualanus Peters, 1883	
	admiralitatum Thomas, 1894	
	pohlei Stein, 1933	
	mariannus Desmarest, 1822	including *loochooensis*
	pelewensis Andersen, 1908	including yapensis
	? *howensis* Troughton, 1931	no molecular data
	? *faunulus* Miller, 1902	no molecular data
group *incertae sedis*	*melanotus* Blyth, 1863	no molecular data
	melanopogon Peters, 1867	no molecular data
	keyensis Peters, 1867	no molecular data
	aruensis Peters, 1867	no molecular data
	argentatus Gray, 1844	no molecular data
	caniceps Gray, 1870	no molecular data
	subniger (Kerr, 1792)*	no molecular data

* Asterisks identify extinct species and question marks identify species whose group placement is not yet supported by molecular data.

manufacturer protocol was slightly changed to increase lysis incubation time: the skin samples were incubated for 24 h in buffer ATL and Proteinase K, then another 20 uL of Proteinase K was added and incubation proceeded for an extra 24 h. For the museum skin samples, we attempted to obtain sequences of two mitochondrial loci, Cytochrome b (Cytb, 1140 bp) and rRNA12S (12S, 1200 bp) genes. PCR amplifications of skin DNA samples were carried out using PCR beads (GE Illustra PureTaq Ready-To-Go PCR beads) and additional reaction cycles. A number of combinations of external and internal primers were used in order to obtain complete sequences (Fig. 1). Some of the primers used were obtained from the literature while others were newly designed based on available *Pteropus* data (Supplementary material Table S4). To avoid contamination, tissue and museum skin DNA samples were kept in separate labs and were never used in the same PCR. Contamination with other *Pteropus* species DNA was checked by confirming that sequences obtained from a museum skin sample were not identical to sequences of another species. Low-quality

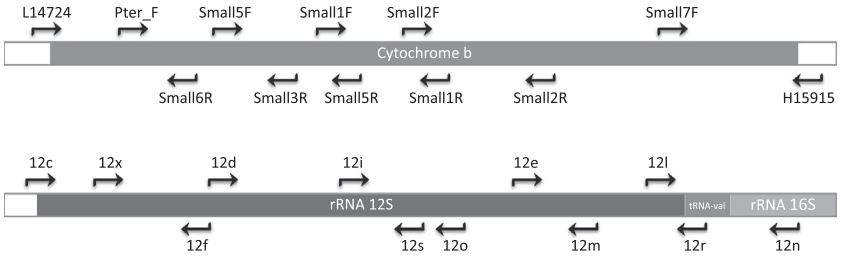

Fig. 1. Primers used to obtain sequences from preserved skin samples. Primer sequences are listed in the Table S4 of the supplementary material.

samples were sequenced several times and dubious nucleotide reads were recorded as unknown in the alignment.

To better address the relationships among *Pteropus* and related genera, we additionally sequenced three nuclear loci from preserved tissue samples: partial Recombination Activating Gene 1 (RAG1, 1084 bp), exon 28 of the von Willebrand Factor gene (vWF, 1231 bp), and partial Breast Cancer 1 gene (BRCA1, 1352 bp). PCR amplification and sequencing of tissue samples was carried out using primer combinations previously published (Almeida et al., 2009, 2011; Giannini et al., 2009). All fragments were sequenced in both directions using an ABI 3730xl DNA Analyzer. The sequences were edited using the software Sequencher 4.2 (Gene Codes).

2.3. Phylogenetic analysis

Sequences were aligned with MAFFT (Katoh et al., 2005). Phylogenetic analyses were performed using PAUP* (Swofford, 2002) for Maximum Parsimony (MP), RAxML (Stamatakis, 2006) for Maximum Likelihood (ML), and MrBayes (Ronquist and Huelsenbeck, 2003) for Bayesian (BI) tree searches. The MP analysis was done with random sequence addition and 1000 replicates, and node support was estimated with Bremer decay index using TreeRot (Sorenson and Franzosa, 2007). For the ML analyses, tree searches were done in 20 replicates and bootstrap values were obtained with 100 replicates. Bayesian tree searches were done in 2 runs, with 10 million generations each, and 4 chains. Parameters were sampled every 1000 generations and their distributions were visualized using Tracer to check for convergence. Conservatively, of the 10,000 sampled trees, the first 5000 were used as burn-in. In both the BI and ML analyses, the GTR + Γ model was applied and the parameters were estimated separately from each partition of the data: entire 12S; nuclear genes, 1st and 2nd codon positions; nuclear genes, 3rd codon position; Cytb, 1st and 2nd codon positions; and Cytb, 3rd codon position.

The analyses involving museum skin sequences were conducted carefully in order to reduce errors due to contamination and sample misidentification, as well as the effects of missing data in cases where only very small fragments were available. For each mitochondrial gene (Cytb and 12S), we first ran separate analyses using all available sequences (tissue, skin, and GenBank) longer than 450 bp to check for obvious unexpected results such as samples of the same species that would not cluster together. In those cases, sequences and sample identification were double-checked and sequences were excluded if still dubious, as was the case for 2 GenBank sequences (AB062473, FJ561378). Then the matrices were reduced to include a maximum of 3 individuals per species with preference given to samples with known collection locality and for which both genes had been successfully sequenced.

An ILD (Incongruence Length Difference) test was subsequently run to check for phylogenetic incongruence between the Cytb and 12S genes in PAUP*, and the results showed the two genes were highly congruent ($p = 1$). The two matrices were then combined, resulting in a single matrix with representative sequences from 43 *Pteropus* species plus 10 other pteropodine species. In the combined analyses of Cytb and 12S, *Melonycteris* was used as outgroup. Sequences shorter than 450 bp, initially excluded to reduce the effects of missing data on tree resolution and clade support, were subsequently added one by one to the matrix. Separate tree searches were then done for each of these new matrices, thus facilitating placement of these short sequences on a robust background phylogeny. Additional phylogenetic analyses were done based on the nuclear gene set alone and the combined mitochondrial + nuclear gene sets. These analyses included only the samples for which nuclear gene sequences were successfully obtained (samples which had DNA extracted from museum skins were not included in those analyses).

2.4. Divergence time estimates

Because *Pteropus* has no known pre-Holocene fossil record, we relied on substitution rates to estimate divergence times. Cytb is the only gene for which an estimate of substitution rate has been obtained based on fossil data for bats: the divergence between *Myotis nattereri* and *M. schaubi* at 6 million years (My) and the divergence between *M. daubentonii* and *M. bechsteinii* at 5 My (Ruedi and Mayer, 2001; Hulva et al., 2004). Therefore, we pruned our matrix leaving only Cytb sequences that were at least 750 bp, with one randomly selected sequence per species. An ML tree was obtained for this dataset using RAxML as described above. Since Cytb has high rates of substitution and tends to be saturated at large phylogenetic distances (Almeida et al., 2009), we kept only samples of the genus *Pteropus* plus *Neopteryx* and *Acerodon* as outgroups. The molecular clock was tested with the program PAML (Yang, 1997, 2007) and was rejected ($p < 0.0001$), leading us to choose a relaxed clock approach. To date the *Pteropus* clades, we relied on Bayesian methods implemented in the program BEAST (Drummond and Rambaut, 2007).

To choose a prior for the *Pteropus* tree root, in this case the split between *Pteropus* and its sister genera, *Neopteryx* and *Acerodon*, we estimated an interval for this divergence time using our nuclear gene matrix. Using previously estimated node ages based on nuclear sequence data and several fossil ages (Teeling et al., 2005; Almeida et al., 2009), we set as a prior for the split between *Nyctimene* and *Melonycteris* a normal distribution with mean age of 25 My and standard deviation of 1.3 on the program BEAST. In this analysis we obtained a 95% confidence interval (CI) for the divergence time between *Acerodon* and *Pteropus* of 6.5–11.3 million years ago (Mya), with 8.8 Mya as the median. Thus, we set the tree root prior of the *Pteropus* Cytb tree as a lognormal distribution with median of 8.8 and standard deviation of 0.2. The prior for the mean substitution rate was set as a lognormal distribution with mean of 0.023 subs/site/My (Ruedi and Mayer, 2001; Hulva et al., 2004) and a standard deviation of 0.4 (95% CI 0.011–0.041). The sequence

data were partitioned into 1st + 2nd and 3rd codon positions and the GTR + Γ + I model was used with parameters to be estimated from the data.

To include a calibration point on the tree, we used island age to set an upper boundary on the divergence time of *P. ualanus*, endemic to Kosrae Island (part of the Caroline Islands, Micronesia), from its sister species *P. admiralitatum* and *P. tonganus*. Kosrae is a volcanic arc island dated in 2.6–1.4 Mya (Keating et al., 1984). We used 2.6 My as the maximum age for the divergence of *P. ualanus* from its sister species. It is important to note here that island age can be very poorly correlated with species divergence time. First, it only gives an upper limit for the presence of that species in the island. Second, a species maybe actually older than the island it currently occupies, having reached it after previous divergence in another island where it has gone extinct (e.g. Thorpe et al., 2005). Therefore, the divergence time analysis was done both with and without this calibration point.

We ran BEAST for 10 million generations and checked for parameters' conversion with Tracer (Rambaut and Drummond, 2003). If conversion was not attained, we reran the analysis for 50 million generations. To test our results, several analyses were performed varying the substitution model (GTR + Γ + I or HKY SRD06 models), using the tree prior for the root, and varying the speciation model (Yule and birth-and-death).

3. Results

3.1. Museum skin sequencing results

Sequences were obtained for 25 samples taken from museum skins of 22 species (Supplementary Table S1). Sequencing success was very high for samples collected during and after the 1970s. For the samples collected before 1970, the length of sequenced fragments was significantly shorter and highly variable (Fig. 2). This variation is probably related to museum conservation considerations such as chemicals used in preparation and exposure to light. The oldest specimen sequenced was collected in 1904 (*P. pselaphon*). Human sequence contamination was minimal and happened with only one primer pair (12Sd + 12So). One instance of contamination with another bat sequence was detected involving two museum samples. These two samples were resequenced and one of them did not yield any sequence the second time, while the other consistently matched the original sequence obtained.

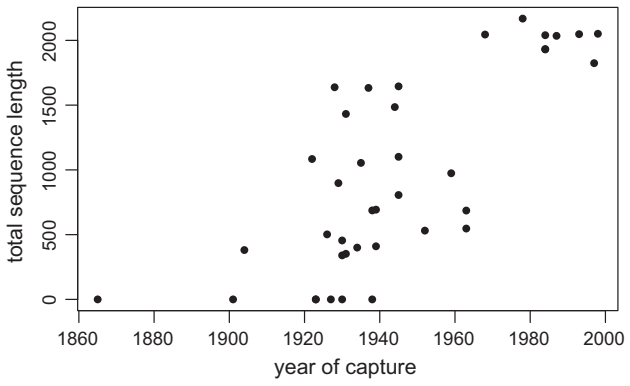

Fig. 2. Total sequence length (cytochrome b + 12SrRNA) obtained as a function of the year of capture of the museum specimens sampled for this study.

3.2. Relationships within the genus Pteropus

The combined 12S + Cytb matrix had 910 variable characters, 785 of which were parsimony informative; these numbers dropped to 730 and 559, respectively, within *Pteropus*. Analyses of this dataset resulted in trees with some highly-supported clades that were also congruent across methods, and a few poorly-supported groupings that were incongruent across trees derived with different methods (Fig. 3, Bayesian and MP trees in the Supplementary material Figs. S1 and S2). Among the well-supported relationships are three large *Pteropus* clades and two isolated, single-species lineages: *P. scapulatus* and *P. lombocensis*.

We then used the ML tree obtained as a scaffold for positioning other *Pteropus* species for which only very small sequence fragments were available: *P. tokudae* (139 bp + 213 bp; [Cytb + 12S]), *P. pselaphon* (167 bp + 214 bp), *P. tuberculatus* (206 bp + 296 bp) and *P. rennelli* (284 bp + 171 bp), for which we were able to obtain small sequences of both the 12S and Cytb genes, and *P. ocularis*, *P. personatus*, *P. temmincki*, and *P. fundatus*, for which relatively conserved fragments of the 12S gene were available from GenBank (Colgan and da Costa, 2002). The relationships of these species were investigated using both ML and MP methods, which agreed in all cases. These analyses allowed us to position 7 more species on the *Pteropus* tree, 4 of which were found to nest within one of the large main clades with good statistical support (these species were known from fragments of both mitochondrial genes). The resulting trees are shown as subsets in Fig. 4. One interesting result was the positioning of *P. personatus* outside the genus *Pteropus*. Although this result lacks statistical support, it suggests that *P. personatus* may not belong within *Pteropus*, and deserves further attention as a potential genus-level lineage within Pteropodini. *Pteropus ocularis* and *P. temmincki* each appeared as additional isolated single-species *Pteropus* lineages, but without significant statistical support (Supplementary material Figs. S3 and S4).

3.3. Relationships within the Pteropodini Tribe

Of the 3664 characters in the nuclear genes-only matrix, only 455 were variable, 263 of which were parsimony informative. The ML tree obtained when this matrix was analyzed showed many similar relationships to those obtained with the mitochondrial data alone, but also some important differences (Fig. 5). Within *Pteropus*, there were lower resolution and lower bootstrap values – likely a consequence of the relatively low variation of these nuclear genes at this taxonomic level – but some of the main clades, including the phylogenetically isolated *P. scapulatus*, were recovered. A noteworthy difference between the nuclear and the mitochondrial trees was in the position of *P. poliocephalus*. In the nuclear tree it was recovered within the "*vampyrus*" group as sister to *P. medius*, while in the mitochondrial tree it was found to belong to a different *Pteropus* clade. Although the discordant clustering of *P. poliocephalus* with Indian Ocean species in the nuclear tree did not have a significant bootstrap percentage, it was supported by an exclusive insertion (3 bp long with identical nucleotides) in the BRCA1 gene. *Neopteryx* and *Acerodon* appeared, similarly to the mitochondrial tree, as sister to *Pteropus*, although the relationships among these three genera could not be resolved with any dataset. Relationships recovered for the remaining genera were also significantly different between the datasets.

The ILD test rejects the hypothesis of congruence in phylogenetic signal ($p < 0.004$) between mitochondrial and nuclear partitions. Nevertheless, the concatenation of the two datasets resulted in a tree not only more resolved, but also with higher statistical support for several clades (Fig. 6). *Pteropus poliocephalus* was excluded from the combined nuclear + mitochondrial dataset

Fig. 3. Maximum likelihood tree based on the concatenated sequences of the cytochrome b and rRNA12S genes. Bootstrap values are shown above branches. Branches in dashed lines represent clades not recovered in the MP and/or the Bayesian trees (both available as supplementary material).

analysis due to the incongruent phylogenetic positions shown with the nuclear and the mitochondrial datasets (see discussion above).

3.4. Divergence time of Pteropus species

The divergence time estimates (Fig. 7) were very stable to alternative sequence evolution and speciation models. The estimated standard deviation of the substitution rate was 0.11, which suggests relative homogeneity of rates. Used as a calibration point, the divergence of *P. ualanus* did not affect the analysis since the estimated date for the divergence of this species from *P. tonganus* (0.6 Mya) was much lower than the maximum island age (2.6 Mya). Exclusion of this prior from the analysis did not change the results.

The age of the last ancestor that *Pteropus* species shared with other pteropodines was estimated at 8.0 My (95% CI 6.6–10.6). The divergence time analysis estimated the first split in *Pteropus* at 6.6 Mya and the most recent split between our sampled taxa at 0.03 Mya (between *P. pelewensis* and *P. yapensis*, which we regard as conspecific—see below). Most *Pteropus* lineages originated after the Early Pliocene and two large speciose clades (the "*vampyrus*" and "*griseus*" groups) diversified during the Pleistocene (Fig. 7). These two bursts of diversification occurred in different regions: one clade includes the Indian Ocean species, while the

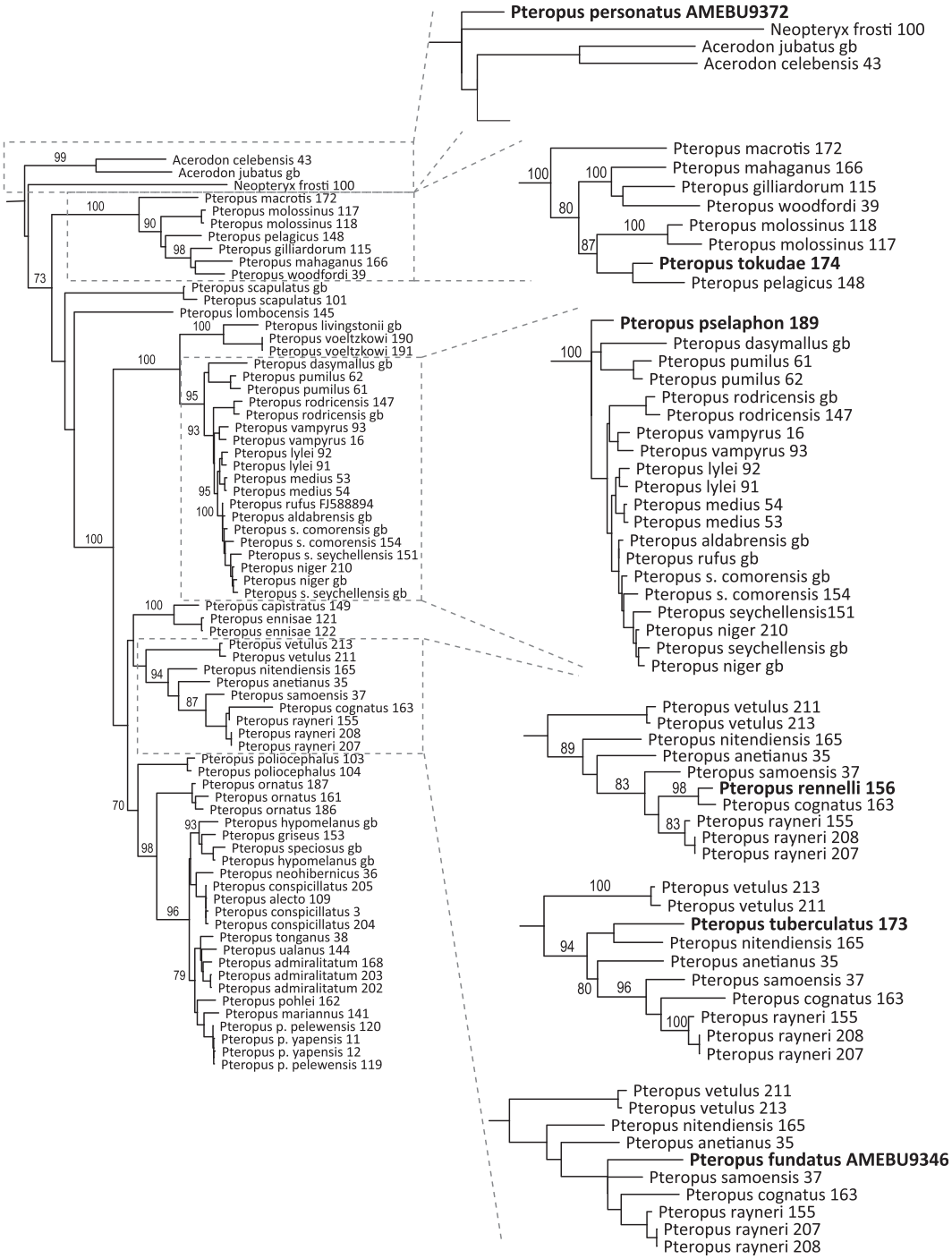

Fig. 4. Phylogenetic position of species with little sequence data as recovered in a maximum likelihood analysis based on the concatenated sequences of the cytochrome b and rRNA12S genes.

other clade is distributed mainly in Melanesia and Micronesian islands, although it also includes species from Australia and insular South East Asia.

4. Discussion

4.1. Relationships within Pteropodini

In addition to *Pteropus*, our study included all other genera of the tribe Pteropodini (Bergmans, 1997). We were able to obtain both mitochondrial and nuclear gene sequences for at least one species per genus. Our comparisons confirm the generic-level distinctiveness of *Styloctenium, Desmalopex, Pteralopex, Mirimiri, Neopteryx*, and *Acerodon* with respect to each other and to *Pteropus* (Helgen, 2005; Giannini et al., 2008). Nevertheless, relationships among genera were not well resolved. There was significant incongruence among datasets, depending upon both taxonomic sampling and markers used. Some relationships were stable across datasets, such as the close relationship between *Acerodon*, *Neopteryx*, and *Pteropus*, although the resolution within this group was also contentious. Expanded molecular datasets are needed to better resolve intergeneric relationships with Pteropodini.

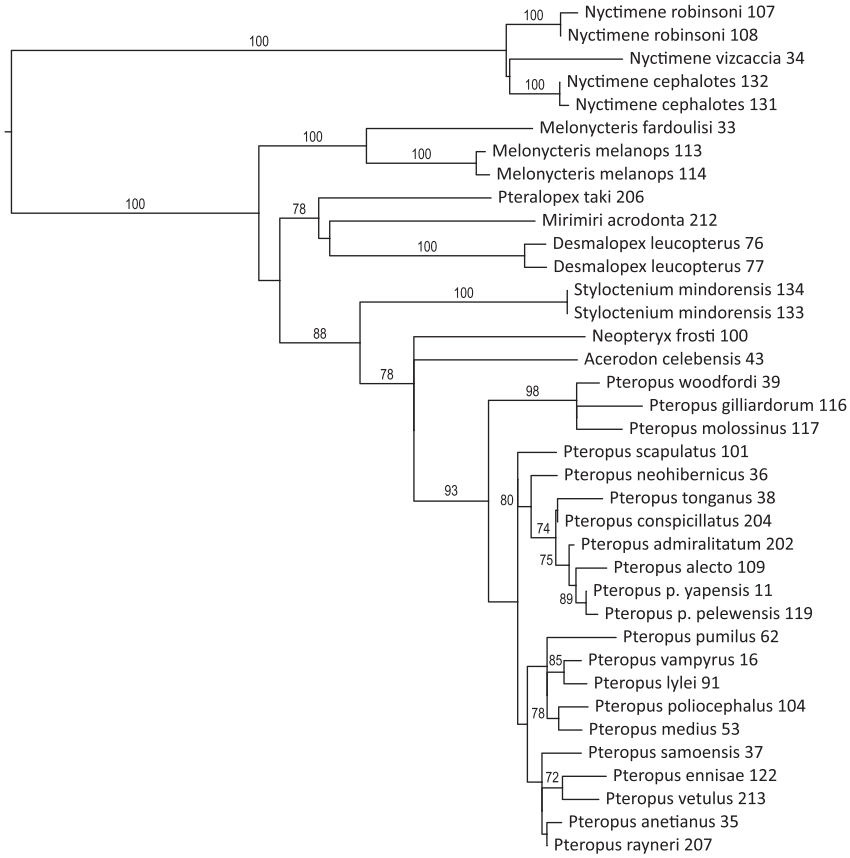

Fig. 5. Maximum likelihood tree based on fragments of three nuclear genes (*RAG1*, *vWF*, and *BRCA1*). Bootstrap values higher than 70% are shown above branches.

4.2. Monophyly of Pteropus

The question of the monophyly of *Pteropus* was recently reviewed by Giannini et al. (2008), who recognized *Desmalopex*, previously regarded as a synonym of *Pteropus*, as a distinct genus comprising Philippine taxa not closely related to *Pteropus* (Esselstyn et al., 2008; Giannini et al., 2008). Our analyses confirm that *Desmalopex* is not particularly closely related to *Pteropus* and is instead probably sister to the monkey-faced bats, *Pteralopex* and *Mirimiri*, and affirm the deep phylogenetic split between the latter two genera (Helgen, 2005). Our results also suggest that one additional lineage likely also belongs outside the genus *Pteropus–P. personatus*, a highly distinctive species. This result, however, is based on limited sequence data consisting only of a relatively conserved, short fragment of the 12S gene obtained from GenBank (published originally by Colgan and da Costa, 2002), and this placement lacks statistical support in our analyses. It will be essential to obtain more sequence data from additional samples of *P. personatus* to test this hypothesis of relationships. With the exception of species now classified in *Desmalopex* and *P. personatus*, all other putative *Pteropus* species included in our sample were recovered together to the exclusion of other pteropodines in our trees, suggesting that we are at last nearing a firm understanding of the monophyletic content of the diverse genus *Pteropus*, an important goal in bat taxonomy.

4.3. Species-level taxonomy

The phylogenetic trees obtained in our study revealed issues regarding species status that deserve further investigation. First, our results suggest a complex taxonomic situation involving *P. hypomelanus* and its closest relatives, *P. griseus* and *P. speciosus*. Specimens of *P. hypomelanus* from Calayan (Philippines) clustered with a specimen of *P. speciosus* from Mindanao (Philippines). These sequences were obtained from GenBank and only the Cytb gene (entire gene sequence) was available for *P. speciosus*. Another specimen of *P. hypomelanus* was sister to a clade formed by the above-mentioned specimens plus *P. griseus* (Fig. 3). Unfortunately, the geographic origin of the non-Philippine specimen of *P. hypomelanus* is not known with certainty; it was either captive-born (at the Lubee Bat Conservancy, Gainesville, Florida) or was collected in Pulau Panjang, an island off the northwest coast of Java (B. Pope, personal communication). One of us (K. Helgen) is involved in a detailed review of the taxonomy of *P. hypomelanus* and close relatives that may resolve this apparent taxonomic complexity.

Another issue involving species limits involves some of the Indian Ocean members of the "*vampyrus*" group. As pointed out previously by O'Brien et al. (2009) and Chan et al. (2011), the remarkable morphological differentiation among some of these taxa (involving many of the typical characters used in *Pteropus* taxonomy such as pelage patterns, craniodental characters, and measurements; see Andersen, 1912; Bergmans, 1990) is not accompanied by deep genetic divergence. Divergence time estimates suggest this clade is less than 0.5 My old, implying that incomplete lineage sorting has likely contributed to the lack of genetic differentiation between these morphologically distinctive taxa that are presently isolated in remote landmasses throughout the western Indian Ocean. Recent evidence suggests that long distance migration (Chan et al., 2011) may be relatively common in this group (e.g., recolonization of Reunión by *P. niger*), suggesting that occasional introgressing hybridization may also play a role in the lack of mitochondrial differentiation in this young clade. Interestingly, the estimated divergence date of *P. aldabrensis* of about 0.100 My (Fig. 7) is in accordance with the estimated date

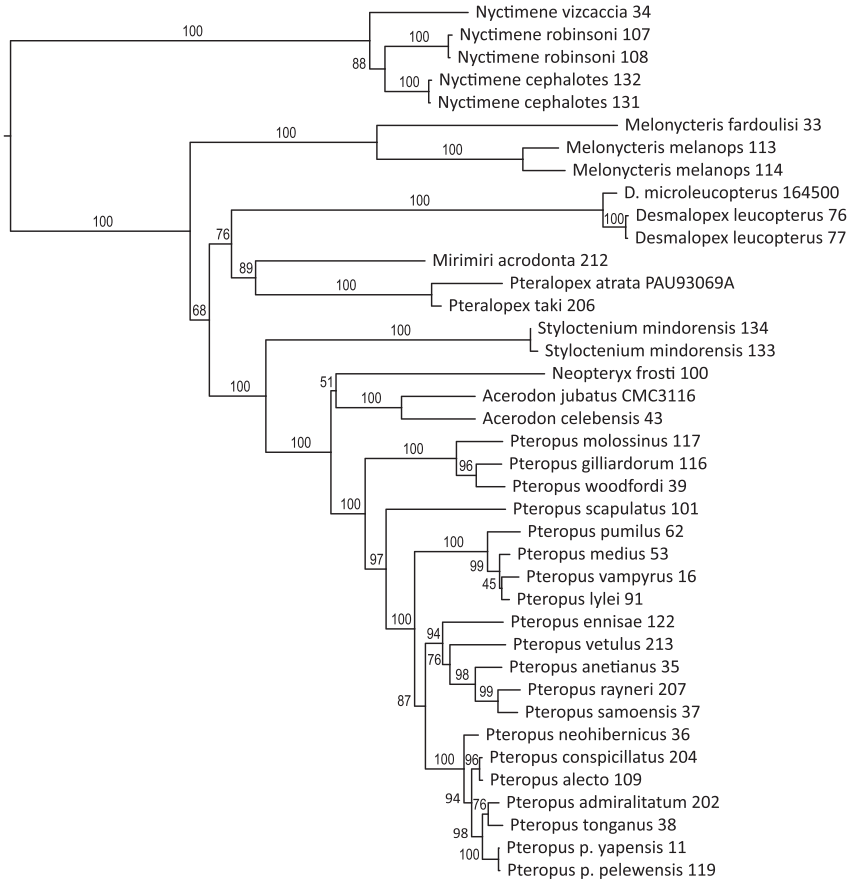

Fig. 6. Maximum likelihood tree based on nuclear (*RAG1*, *vWF*, and *BRCA1*) and mitochondrial genes (*Cytb* and *rRNA12S*). Bootstrap values are shown above branches.

of the emergence of Aldabra atoll dates (0.125 My; Warren et al., 2005).

In two other cases, molecular divergence between species was extremely low. The first case involves two species with endemic distributions on islands that are about 400 km apart: *P. yapensis* from Yap and *P. pelewensis* from Palau. Among the two *P. yapensis* and the two *P. pelewensis* individuals sampled, for which the entire Cytb gene sequence was obtained, only 2 substitutions were found, both in third codon position sites. This amount of substitution (0.1%) is more consistent with variation among samples within a species than variation between species (Lim et al., 2004; Bradley and Baker, 2001). The 12S gene was sequenced entirely in samples from both islands and showed similar results. Divergence time dating suggests their Cytb sequences diverged only about 30 thousand years ago. We have examined most specimens of *pelewensis* and *yapensis* (including the nominal taxon *ulithiensis* from Ulithi Atoll) in world museums (Supplementary material Table S5)—the two taxa are very similar, differing only in average size (e.g., condylobasal length averaging 53.4 mm in *pelewensis* [range 50.9–56.7; $n = 19$], and 57.3 mm in *yapensis* [54.0–61.0; $n = 31$]). We conclude that they are best regarded as conspecific, and we here designate *P. pelewensis* Andersen, 1908 (which has page priority over *P. yapensis* Andersen, 1908) as the name to be used (we recognize the subspecies *P. p. pelewensis* of Palau and *P. p. yapensis* of Yap and Ulithi). Other authors have suggested that these taxa may be conspecific with the remote Pacific flying foxes *P. mariannus* (Guam and the Mariana Islands) and *P. ualanus* (Kosrae) (Mickleburgh et al., 1992; Pierson and Rainey, 1992; Wiles and Brooke, 2009) but primary systematic studies have been lacking. Our genetic comparisons and museum examinations suggest that these latter taxa, which can also be more clearly distinguished from *P. pelewensis* morphologically, are better regarded as distinct species. Brown et al. (2011) studied populations of *P. mariannus* and *P. p. pelewensis* from several islands using both mitochondrial gene sequences and nuclear microsatellites. Although their analyses confirmed the distinctiveness of *P. p. pelewensis* with both types of markers, they did not find support for the genetic distinctness of *P. mariannus* subspecies within the Marianas archipelago.

The second case involves *P. conspicillatus* (represented in our study by 3 specimens from Papua New Guinea) and *P. alecto* (one specimen from Australia). These species were represented by long sequences (>2000 bp) in our analysis and yet were found to be paraphyletic with our *P. alecto* sample nesting within *P. conspicillatus* (Fig. 3, Supplementary material Figs. S1 and S2). These two species are strongly differentiated morphologically, and can be easily distinguished by their fur coloration and craniodental anatomy. Paraphyly between the two species had previously been observed in a study that employed D-loop (mitochondrial) sequences and several specimens of the two species (Fox, 2006). This phylogenetic pattern can be interpreted either as incomplete lineage sorting of alleles between sister species, or as evidence of hybridization. Although the former process cannot be ruled out, we suggest that hybridization is the most likely explanation. *P. alecto* and *P. conspicillatus* overlap extensively in their ranges in eastern Australia (and likely in southern New Guinea) and can roost together in the same area (Parsons et al., 2010). Interestingly, there is evidence that *P. alecto* can interbreed with *P. poliocephalus* (Webb and Tidemann, 1995), to which it is not particularly closely related. Assuming introgression of mitochondrial DNA from *P. conspicillatus* to *P. alecto* or vice-versa, our mitochondrial tree may not illustrate the true evolutionary history for these two species. Unfortunately, the nuclear loci for which we had data show little

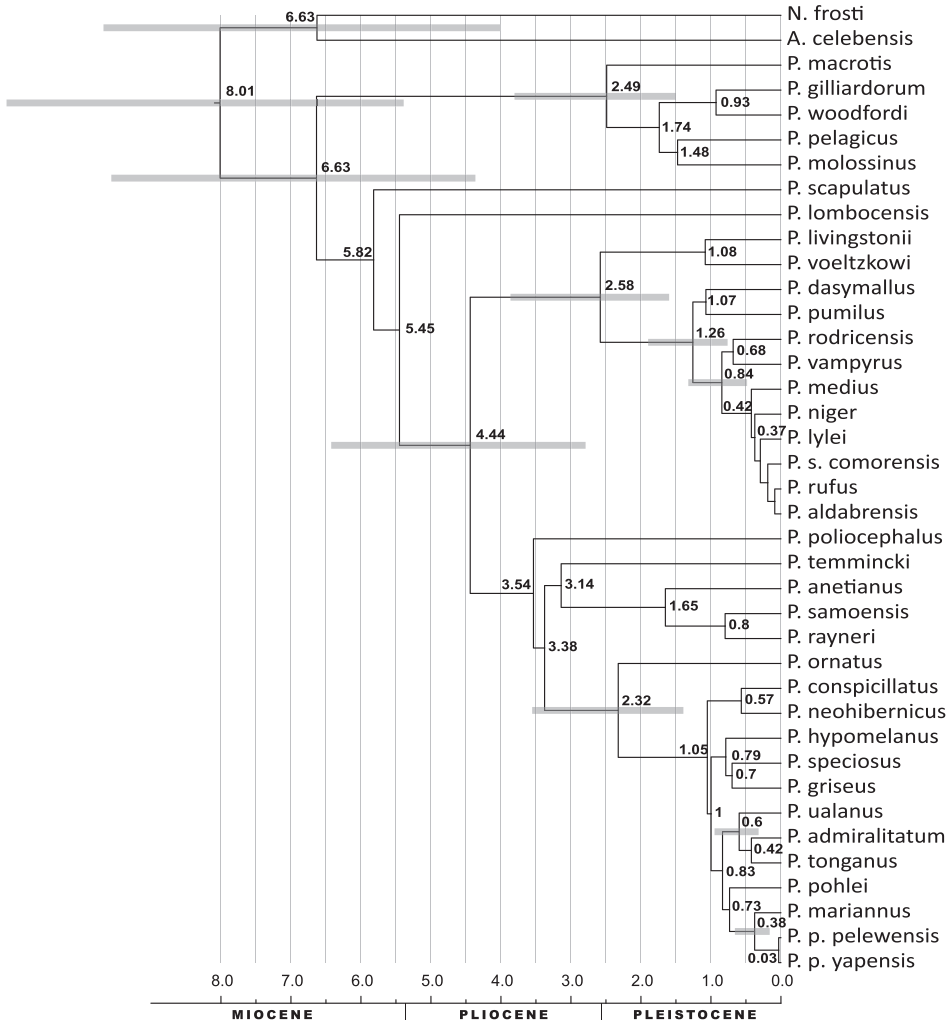

Fig. 7. Dated cytochrome b tree. Numbers on nodes represent the medians of divergence date estimates and the bars represent the 95% confidence interval of the estimates for a sample of the nodes.

variation at the genus level and lack sufficient phylogenetic information to resolve relationships between closely related *Pteropus* species.

In contrast to the above cases suggesting introgression or closer relationship among lineages than previously supposed, our analyses also uncovered a case that indicates greater species diversity than currently recognized. Our sampling of Northern Melanesian flying foxes includes both recognized subspecies of *P. capistratus*, *P. c. capistratus* from New Britain and *P. c. ennisae* from New Ireland. These taxa differ remarkably in that *capistratus* has a striking white and dark face mask, while *ennisae* does not (Flannery and White, 1991; Flannery, 1995). The genetic divergence between *capistratus* and *ennisae* is more characteristic of interspecific rather than intraspecific variation in *Pteropus* (4.7% sequence divergence in Cytb), and based on the morphological and genetic distinction between these two taxa, we suggest that they are best regarded as sister species, *P. capistratus* and *P. ennisae*.

Many other species of *Pteropus* have described subspecies (Simmons, 2005) that were not sampled for this study. Moreover, several species occur in two or more remote islands (or archipelagos) and could present important genetic discontinuities. A more complete geographic sampling of *Pteropus* is needed to solve the specific status and taxonomy of widespread species of *Pteropus*, many of which are locally threatened.

4.4. "Species groups" in Pteropus

Our study included samples from 15 of the 18 traditional *Pteropus* species groups (sensu Simmons, 2005, who followed Koopman (1993) and Andersen (1912)). We were not able to obtain samples of species from three of Andersen's (1912) groups ("*caniceps*" group [*caniceps, argentatus*], "*melanopogon*" group [*melanopogon, keyensis, aruensis*], and "*melanotus*" group [*melanotus*]) (Table 1). Our analyses found that none of the polytypic Andersen (1912) species groups that we sampled are monophyletic. We propose a new classification of *Pteropus* species that reflects the phylogenetic relationships recovered in our trees. In our new classification, *Pteropus* species are split into 13 species groups, 7 of which are monotypic. Many of these groups are united by characteristic morphological features, although there are often exceptions within groups, or similarities between groups. In total, fourteen species (including 5 extinct species) were not included in our molecular comparisons. Some of these species bear close enough morphological resemblance to sampled species that we have tentatively assigned them to our species group framework, but for 8 species we have not attempted to include them in our classification due to lack of any clear knowledge on their phylogenetic relationships (Table 1). In delineating species groups, we use the name of the earliest described species within each group to

denote the species group (e.g., the "*pelagicus*" group, "*vampyrus*" group, etc.).

The most basal split within our taxonomic sampling of *Pteropus* is between a well-supported clade of gracile, small-toothed flying foxes of Melanesia and Micronesia (*P. macrotis*, *P. gilliardorum*, *P. mahaganus*, and *P. woodfordi* of Melanesia, and *P. molossinus*, *P. pelagicus*, and *P. tokudae* of Micronesia) and all other *Pteropus* species. Most (but not all) of these small-toothed species are known or suspected to have diets including substantial percentages of nectar, pollen, and inflorescences as opposed to fruits *per se* (Flannery, 1995; Bonaccorso, 1998; Buden et al., 2013). Andersen (1912) included the members of this group in many different species groups, but we here classify these species as the "*pelagicus*" group. More detailed study of the biogeographic diversification in this clade is needed, but our results suggest the possibility of monophyletic Papuan (*P. macrotis*), Northern Melanesian (*P. woodfordi*, *P. mahaganus*, *P. gilliardorum*) and Micronesian (*P. molossinus, P. pelagicus, P. tokudae*) lineages within this group.

Apart from the "*pelagicus*" group, our analyses recovered two other successive and especially deep divergences in *Pteropus*, one representing the single species *P. scapulatus* (endemic to Australia and southern New Guinea) and the other representing the single species *P. lombocensis* (endemic to the Lesser Sunda Islands, from Lombok to Timor). We propose recognizing separate species groups for each of these two single-species lineages, the "*scapulatus*" group and "*lombocensis*" group, respectively. In terms of general morphology, *P. scapulatus* closely resembles species of the "*pelagicus*" group in its gracile skull and reduced dentition. This apparently reflects similarities in diet; *P. scapulatus* feeds largely on nectar and pollen from *Eucalyptus* blossoms (Birt, 2005). Although *P. lombocensis* was represented in our phylogeny by a single museum specimen collected in 1978 (Goodwin, 1979), most of the Cytb and 12S genes could be sequenced. No nuclear sequences were available for this species, however, to confirm its position on the *Pteropus* tree, and further study of its phylogenetic position is needed. Andersen (1912) placed several other small flying-foxes in his "*lombocensis*" group including *P. molossinus* (which we place in the "*pelagicus*" group) and *P. rodricensis* (which we include in the "*vampyrus*" group; Table 1). Another species that could, similarly to *P. scapulatus* and *P. lombocensis*, be in its own species group is *P. ocularis*. This suggestion, however, is based on a small fragment of the 12S gene and lacks statistical support; hence we consider the species-group assignment of *P. ocularis* to be uncertain.

A clade recovered in all analyses with high support was one that groups all the western Indian Ocean species with related Asian species (*P. vampyrus, P. medius, P. lylei*). The phylogeny and biogeography of this clade has been analyzed and discussed in detail by O'Brien et al. (2009) (see also Chan et al., 2011). Here we increased sampling within this clade to include *P. lylei* and *P. pselaphon*. We propose classifying the species of this clade into two species groups: the "*livingstonii*" group for the Indian Ocean/East African taxa *P. livingstonii* and *P. voeltzkowi*, and the "*vampyrus*" group for the remaining species. As discussed by O'Brien et al. (2009) and confirmed by our divergence time estimates, the "*vampyrus*" group has undergone a recent explosive diversification on a timescale less than 1 Mya.

The third and last of the principal clades is comprised of Pacific Ocean and Australasian species, including the widespread *P. hypomelanus*, which represents an incompletely understood complex of species. This clade was consistently recovered, though it was not as highly supported as the other principal clades. In this clade, internal relationships, especially those at its base, were not well resolved, showing different arrangements depending on the dataset and the tree-search method used. Most trees show that this clade has an early subdivision into three subclades, one of which is represented by a single species, *P. poliocephalus*. We propose that *P. poliocephalus*, which is morphologically distinctive (in terms of craniodental anatomy) within this clade, be recognized as a monotypic "*poliocephalus*" group.

The second subclade in the large Pacific/Australasian clade can be divided into three species groups: the "*capistratus*" group, the "*vetulus*" group, and the "*samoensis*" group. The "*capistratus*" group includes two sampled species, *P. capistratus* and *P. ennisae*, previously considered conspecific subspecies (see above). These taxa are both distributed in the Bismarck Archipelago to the east of New Guinea. *Pteropus temmincki* from the Central Moluccas (Buru, Ambon, and Seram) has traditionally been considered closely related to these species, and Andersen (1912) united them in a single species group. Both species are small, pale-colored flying foxes that appear to associate in small numbers rather than in larger roosting groups, and may be most common in medium-elevation forests rather than coastal lowlands, unlike most other flying foxes. Unfortunately, the only sequence available for *P. temmincki* was a small fragment of the 12S gene, and the placement of this taxon in our trees was inconclusive.

Andersen (1912) and subsequent authors also included *P. personatus* in the "*capistratus*" group. *Pteropus personatus* is a small, brightly-colored species with striking facial coloration. It is the smallest species of *Pteropus*, and its molar dentition, particularly the last molars, is reduced relative to those of other *Pteropus* species. The limited sequence data available for *P. personatus* suggest that, like other small Wallacean pteropodines with striking face-mask coloration (*Neopteryx, Styloctenium*), *P. personatus* may belong outside the phylogenetic scope of *Pteropus*. Additional sequence data is necessary to verify this result.

The "*vetulus*" group is monotypic, including only the small, highly distinctive *P. vetulus*, endemic to New Caledonia. Andersen (1912) was unable to study specimens of *P. vetulus*, so could not allocate it to a species group. Citing its small size, dark coloration, and cuspidate molars, Flannery (1995) regarded it as a highly distinctive, morphologically isolated species.

The "*samoensis*" group consists of eight species included in our analyses. Species of the "*samoensis*" group are endemic to the south-west Pacific (Solomon Islands, Vanuatu, Fiji, Samoa). Most of these species are characterized by very strong jaws and teeth, though one species, *P. fundatus*, is smaller and relatively more gracile than the others. The group was named after *P. samoensis*, which is represented herein by the subspecies *P. s. nawensis* from Fiji; the other recognized subspecies is *P. s. samoensis* from Samoa (Helgen et al., 2009). Though not included in this study, the recently described *P. coxi* of Samoa (very large jaws and teeth) is presumed to be part of this species group, and the much smaller *P. allenorum*, also a recently described Samoan endemic species with similarities to *P. fundatus*, may be the most gracile member of this clade (Helgen et al., 2009). The enigmatic Australian taxon *P. brunneus* very closely resembles *P. cognatus* and *P. rennelli* and likely also belongs in this group. *Pteropus coxi*, *P. allenorum,* and *P. brunneus* are all considered recently extinct and known by only 1–2 museum specimens each (Helgen et al. 2009). *Pteropus cognatus* and *P. rennelli*, which differ in color and size, are very closely related and may be better classified as conspecific subspecies, but a critical revision of this group is lacking.

The third subclade of the Pacific/Australasian clade has a basal split separating *P. ornatus* from a large, speciose clade. *Pteropus ornatus*, endemic to New Caledonia, is isolated on a long phylogenetic branch, and we suggest that it deserves its own species group (Table 1). For the remainder of this large clade, the distribution of which spans essentially the entire Indo-Pacific region, we use the name "*griseus*" group. This clade comprises a taxonomically and morphologically diverse group of at least 12 species of medium-sized and large flying foxes (Table 1). The members of this group exhibit many distinctive morphologies and many examples of

sympatric co-occurrences, despite low levels of genetic divergence. Amazingly, the diversification of this speciose group apparently took place during the last 1.1 My. This case of explosive diversification makes this group an ideal system for further and more in-depth studies of diversification and speciation.

4.5. Evolutionary biology of Pteropus

The great diversity of *Pteropus* can be largely explained by its being a specialized island taxon. Islands provide isolated areas (allopatry) where divergence can proceed quickly by genetic drift without interference from frequent gene flow (Mayr, 1942). Small and isolated islands also favor rapid morphological divergence triggered by founder effects and differential ecological adaptation (Thorpe et al., 2010). High morphological diversity and frequent convergence in *Pteropus* is consistent with a hypothesis of adaptive divergence. Extreme vagility in some species, in turn, may favor the dispersion of successful, genetically well-established lineages producing repeated patterns of island invasion. According to our phylogenetic trees, sympatry of *Pteropus* species most often results from multiple colonization events rather than *in situ* speciation. In rare cases where two closely related species occupy the same island or archipelago, these are relatively large landmasses, with ample opportunities for differentiation in allopatry/parapatry (e.g., *P. neohibernicus* and *P. conspicillatus* in New Guinea, *P. mahaganus* and *P. woodfordi* in the Solomon Islands).

Multiple colonization of the same area, resulting in sympatry of distantly related species, has seemingly occurred even in very isolated islands such as Guam (*P. mariannus* and *P. tokudae*), New Caledonia (*P. ornatus* and *P. vetulus*), Samoa (*P. tonganus* and *P. samoensis*), or the Comoros (*P. livingstonii* and *P. seychellensis comorensis*). In all cases there are ecological differences between the sympatric species, especially involving body size, food habits, habitats utilized, or activity periods (Nowak, 1994; Norberg et al., 2000; Helgen et al., 2009), pointing to a role of particular assembly rules that allow ecologically distinct species of diverse origin to coexist in small islands. Such a pattern of ecomorphological differentiation, for instance, was found among species of the genus *Cynopterus* (Pteropodidae) inhabiting the Malay Peninsula (Campbell et al., 2007). Other characteristics that are likely to play important roles in the assembly of these *Pteropus* communities, as well as in the speciation/extinction dynamics of *Pteropus*, are vagility, population density, and vulnerability to local extinction.

We detected one case of strong incongruence between the signal contained in nuclear and mitochondrial genes in *Pteropus*, in this case concerning the relationships of one species, *P. poliocephalus*. Incongruence among genes or organelles may be explained by two main processes: incomplete lineage sorting or hybridization (Pamilo and Nei, 1988; Maddison, 1997; Shaw, 2002). Although there is no simple way to distinguish between these two alternatives, incomplete lineage sorting is more commonly observed between recently diverged species (Maddison, 1997; Joly et al., 2009). *P. poliocephalus* was not found to be closely related to any other species in our study, suggesting that incomplete lineage sorting may not be an appropriate explanation in this instance. The incongruent relationships of *P. poliocephalus* may be better explained by an ancient introgression of mitochondrial DNA of an unsampled (or extinct) species into a taxon related to the "*vampyrus*" group clade. Histories of hybridization may also explain the high similarity in mitochondrial sequences between morphologically well-differentiated species, such as the case of *P. conspicillatus* and *P. alecto*, and *P. seychellensis* and *P. niger*. Many species of *Pteropus* are known to have strong long-distance dispersal potential (e.g. Epstein et al., 2008; Breed et al., 2010; Chan et al., 2011; Roberts et al., 2012), and occasional gene flow between species following extralimital dispersal events may be relatively common, perhaps especially in clades that seem to have experienced recent and explosive diversification. A better understanding of the role played by incomplete lineage sorting and hybridization in the genus will be possible when a more complete taxonomic sampling of nuclear loci with more nucleotide variation is available. Also, studies involving multiple samples of the same species (e.g. Chan et al., 2011; Brown et al., 2011) are essential to understanding lineage sorting through the coalescent process.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.03.009.

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